

# Novel methods for disinfection of prion-contaminated medical devices

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## Summary

**Background** The unique resistance of prions to classic methods of decontamination, and evidence that prion diseases can be transmitted iatrogenically by medical devices pose a serious infection control challenge to health-care facilities. In view of the widespread tissue distribution of the variant Creutzfeldt-Jakob disease agent in human beings, new practicable decontamination procedures are urgently needed.

**Methods** We adapted an in-vivo method using stainless steel wires contaminated with prions to the hamster-adapted scrapie strain 263K. A new in-vitro protocol of surface contamination compatible with subsequent biochemical detection of PrP<sup>sc</sup> (protease-resistant form of the prion protein) from the treated surface was developed to explore the mechanisms of action of methods of decontamination under test. These models were used to investigate the effectiveness of innovative physical and chemical methods of prion inactivation.

**Findings** Standard chemical decontamination methods (NaOH 1N, NaOCl 20 000 ppm) and autoclaving in water at 134°C reduced infectivity by >5.6 log<sup>10</sup> lethal doses; autoclaving without immersion was somewhat less effective (4–4.5 log reduction). Three milder treatments, including a phenolic disinfectant, an alkaline cleaner, and the combination of an enzymatic cleaner and vaporised hydrogen peroxide (VHP) were also effective. VHP alone, which can be compatible with electronic components, achieved an approximately 4.5 log reduction in infectivity (equivalent to autoclaving without water immersion).

**Interpretation** New decontamination procedures are proposed to ensure the safety of medical and surgical instruments as well as surfaces that cannot withstand the currently recommended prion inactivation procedures.

## Introduction

The occurrence in the UK in 1996 of variant Creutzfeldt-Jakob disease (vCJD), linked to the consumption of bovine spongiform encephalopathy (BSE)-tainted meat products, raised concerns that human beings might have been exposed to secondary infections by the BSE/vCJD agent via medical procedures or the administration of human derived biological products, including blood. Many peripheral tissues from patients with vCJD have been shown to be infectious, and by contrast with sporadic Creutzfeldt-Jakob disease (sCJD), the biochemical marker of prion diseases (PrP<sup>sc</sup>, the protease-resistant, pathological form of the prion protein) is detectable in lymphoid organs like spleen, tonsils, thymus, and appendix.<sup>1,2</sup> We have also shown that PrP<sup>sc</sup> is present in the mucosa of the intestine and in peripheral nerves in a non-human primate model of vCJD.<sup>3</sup> The iatrogenic risk of vCJD has recently been substantiated by the report of a probable case of transfusion-related disease.<sup>4</sup>

As a consequence, precautionary measures have been implemented with regard to blood products, tissue grafts, and the decontamination of surgical instruments. Several iatrogenic transmissions of sCJD due to neurosurgical instrument and electrode contamination have been identified in the past.<sup>5,6</sup> To avoid such events in the future, regulatory measures have been taken concerning the re-use and decontamination of

neurosurgical instruments.<sup>7–9</sup> These measures consist mainly of recommendations to use disposable instruments or harsh decontamination procedures—ie, immersion in 1N NaOH for 1 h followed by porous load autoclaving at 134°C for 18 min.<sup>9</sup> Similar measures should, if practicable, be taken for all surgical instruments and for endoscopic devices, but are hindered by the corrosive effect of either NaOH or NaOCl and the incompatibility of autoclaving with all devices containing plastic, gum, joints, or electronic components.<sup>10</sup>

There is thus an urgent need to explore new procedures and chemical formulations that are both effective and practical for use on instruments and surfaces. So far, most inactivation studies have used residues from tested materials as inocula in standard infectivity bioassays,<sup>7,8</sup> although a strong correlation between infectivity and PrP<sup>sc</sup> has been documented in a study of Cohn-fractionated plasma.<sup>11</sup>

A system based on the use of steel wires has been previously described that mimics the contact of surgical instruments in living organisms.<sup>12,13</sup> We applied this method to inactivation studies on prions bound to surfaces. We tested several decontamination procedures and chemical formulations, and devised three new inactivation protocols applicable to fragile devices and surfaces that were equal to or better than autoclaving at 134°C for 18 min.

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## Methods

### Animals

6-week-old female Syrian golden hamsters (Charles River, France) were used in this study.<sup>14,15</sup> All animals were housed in level 3 care facilities officially registered for prion experimental studies on rodents (agreement number A 92-032-02 for animal care facilities, agreement number 92-189 for animal experimentation).

### Infectious material

The hamster-adapted scrapie strain 263K was stabilised and propagated in the Syrian golden hamster.<sup>14,15</sup> Brains of hamsters at the terminal stage of the disease, typically titrating  $1 \times 10^9$  to  $1 \times 10^{11}$  mean lethal doses ( $LD_{50}$ ) per gram, were homogenised at 10% weight/volume in PBS solution. Healthy hamster brain homogenate was used for the negative controls.

### Contamination of surfaces

Stainless steel wires were used for bioassays. Wires (316 stainless steel, 5.0 mm length  $\times$  0.16 mm diameter) were cleaned by ultrasonication in a 2% Triton X-100 solution for 15 min, rinsed in distilled water, and dried at 37°C for 1 h. The wires were artificially contaminated by immersion in normal 10% brain homogenate (negative controls) or scrapie brain homogenate (positive controls) for 1 h at room temperature. Wires were then dried for 16 h at room temperature. Excess of unbound infectivity was removed by rinsing for 5 min in PBS. To establish an end-point titration and a dose-incubation period curve, wires were immersed in serial 10-fold dilutions of positive homogenate in negative homogenate. To assess the effect of rinsing before implantation, additional control wires ( $1 \times 10^9$  and  $1 \times 10^5$  dilutions) were rinsed for 15 min in PBS after contamination and drying. For in-vitro experiments, glass slides instead of stainless steel wires were contaminated with 20  $\mu$ L of 10% brain homogenate and dried at 37°C for 1 h.

### Decontamination methods

All decontamination methods were done on three independent batches of 5 contaminated wires. Wires were then rinsed in 1 mL of distilled water, dried and stored at -80°C before inoculation. For in-vitro studies, contaminated glass slides were used and decontaminated according to the same protocols.

Control treatments recommended by WHO included:<sup>9</sup> immersion in NaOCl (freshly prepared solution at 20 000 ppm, 1 h, 20°C), in NaOH (1N, 1 h, 20°C), or autoclaving in a porous load cycle (18 min, 134°C). Decontamination methods under test included autoclaving wires immersed in water (18 min, 134°C), immersions in an enzymatic solution (Klenzyme, STERIS, 0.8% v/v in water, 5 min, 43°C), an alkaline cleaner (HAMO 100 Prion Inactivating Detergent, STERIS, 1.6% v/v, 15 min, 43°C), formulated peracetic

acid (STERIS 20 at use dilution, 12 min, 55°C) or phenol disinfectants (Environ LpH or LpHse, STERIS, 5% v/v, 30 min, 20°C). In parallel, batches of wires were treated with the enzymatic cleaner (as above) followed by immersion in water and autoclaving at 121°C for 20 min. Exposures to vaporised hydrogen peroxide (VHP) were done in a sealed container directly coupled to a VHP1000 Biodecontamination System.<sup>16</sup> The latter generated and maintained a dry (non-condensing) hydrogen peroxide gas at a concentration of 1.0 to 1.5 mg/L, for 3 h at about 25°C. Wires were exposed to VHP with or without previous treatment with an enzymatic cleaner.

### Bioassays

Wires were individually implanted into the prefrontal subcortical region of anaesthetised hamsters. Animals were regularly monitored for clinical signs of transmissible spongiform encephalopathy (TSE), and killed at the terminal stage of the disease.  $LD_{50}$  values were determined according to Reed and Muench's method.<sup>17</sup> Diagnosis of TSE was confirmed by detection of PrP<sup>res</sup> in brain by ELISA and western blot techniques, according to a previously described protocol.<sup>18</sup>

### In-vitro analysis of mechanisms of action

For in-vitro analysis, the dried inoculum was treated, and, after removal from glass slides, resuspended in 120  $\mu$ L of water. Samples were treated or not with increasing amounts of proteinase K for 10 min at 37°C, 2 $\times$  Laemmli buffer was added, and 20  $\mu$ L of each extract was used for PrP<sup>res</sup> detection.<sup>19</sup>

In the cases of LpH and LpHse, the product was added 4:1 to 20% brain homogenate in 5% glucose solution and incubated for 30 min at 20°C. Since phenol inhibits proteases, we had to extract PrP<sup>res</sup> from the phenolic phase using an SAF protocol (previously described omitting the proteinase K treatment, from a commercial kit Bio-Rad).<sup>20</sup> Samples were then treated with increasing amounts of proteinase K. A sample corresponding to 200  $\mu$ g of brain was loaded on a SDS-page gel (12% polyacrylamide) and electroblotted onto a PVDF membrane. Immunoblotting was done using a mouse monoclonal antibody (SAF-60, raised against hamster PrP, codon 142-160) followed by a peroxidase-conjugated goat anti-mouse antibody (Southern Biotechnology Associates, Birmingham AL, USA). Immunoreactivity was visualised by chemiluminescence (ECL, Amersham, Orsay, France) and detected by standard autoradiography.

### Immunohistochemistry

Immunohistochemistry analyses were done as previously described.<sup>1</sup> Each brain containing the inserted wire was fixed by immersion in Carnoy's fluid, and then transferred to butanol until paraffin embedding with removal of the wire before microtome section.

	Transmission rate (%)	Total death / total number	Disease in days
<b>Positive control duration(dilution)</b>			
1×10 <sup>-1</sup>	100%	12/12	90 (2)*
1×10 <sup>-2</sup>	100%	12/12	98 (5)*
1×10 <sup>-3</sup>	100%	12/12	117 (6)*
1×10 <sup>-4</sup>	100%	12/12	124 (11)*
1×10 <sup>-5</sup>	92%	11/12	201 (60)*
1×10 <sup>-6</sup>	22%	2/9	201 (39)*
1×10 <sup>-7</sup>	0%	0/12	>365
1×10 <sup>-8</sup>	0%	0/12	>365
1×10 <sup>-9</sup>	0%	0/12	>365
1×10 <sup>-3</sup> (PBS)†	92%	11/12	114 (5)*
1×10 <sup>-5</sup> (PBS)†	46%	4/11	166 (43)*
<b>Negative control (dilution)</b>			
1×10 <sup>-1</sup>	0%	0/12	>365

Data are mean (SE). \*Wires were rinsed in PBS for 15 min before inoculation.

**Table 1: Infectivity of wires exposed to ten-fold serial dilutions of 263K strain hamster scrapie brain homogenates**

5-µm-thick sections were cut and mounted on poly-lysine-coated slides. After treatment with proteinase K (2 µg/mL, 10 mins at 37°C), PrP was detected with a monoclonal antibody coupled to biotine (SAF-32, raised against hamster PrP, codon 79–92, 1 µg/mL, 2 h at room temperature).

### Role of the funding source

This work has been partly supported (in-vivo studies) by Steris Ltd. Steris had no role in the collection and analysis of data, and the role of Steris in study design was limited to protocols of wire decontamination.

### Results

Wire contamination with serial dilutions of brain homogenates from hamsters terminally ill with scrapie (strain 263K), and implantation into recipient animals, established a relation between the infectivity titre (endpoint at 1×10<sup>-6</sup> LD<sub>50</sub>), the transmission rate, and the incubation period (table 1). The progressive decrease in the infection rate, along with an increase in the incubation period as the dilution of the infective material increases, is similar to previous findings with direct inoculation of brain homogenate.<sup>15</sup> Implantation of two groups of wires rinsed for 15 min in PBS led to a slightly lower attack rate in the bioassay when compared with the unrinsed wires, with no prolongation of the incubation period.

Our results are summarised in table 2. The reductions of infectivity were obtained by comparing the attack rate and incubation periods of the transmissions shown in table 2 with those of the dilution series shown in table 1. All treatments producing an infectivity reduction of 5.6 logs or more (corresponding to the threshold of infectivity detection) are considered as having produced complete decontamination within the limit of sensitivity of our model.

Both WHO-recommended chemical treatments (NaOH 1N and NaOCl 20000 ppm)<sup>9</sup> led to a complete decontamination. However, inactivation by autoclaving at 134°C for 18 min was incomplete unless the wires were immersed in water during the autoclaving cycles. The enzymatic cleaner resulted in partial decontamination, but was greatly enhanced by combination with autoclaving at 121°C. VHP by itself reduced infectivity by about 4.5 logs, but resulted in complete decontamination when combined with the enzymatic cleaner. The alkaline cleaner achieved full disinfection at the recommended temperature, as did the phenolic disinfectant Environ LpH (previously known as LpH, which had been shown to be effective in suspension studies<sup>21</sup>).

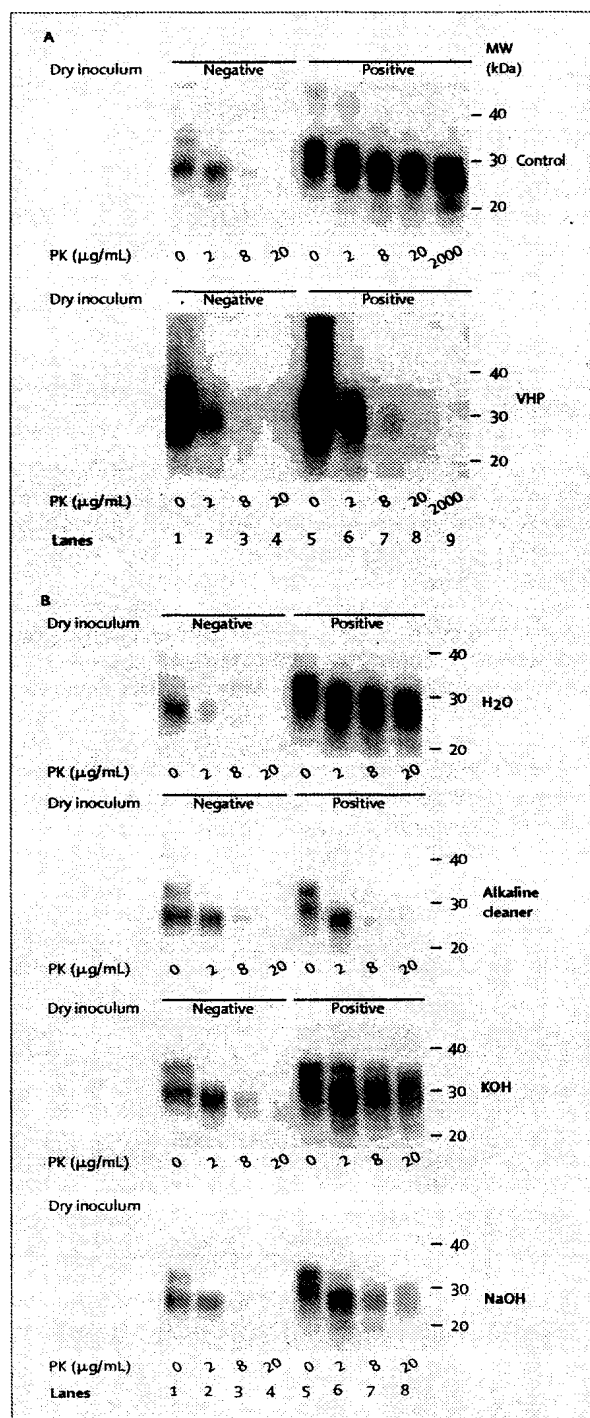
The effects of treatments on the amount of PrP<sup>res</sup> and its degree of protease resistance were investigated by western blots using SAF-60 monoclonal antibody (similar results were obtained with other antibodies of different specificities; data not shown). To reproduce a surface contamination and decontamination, the treatments were applied on glass slides previously contaminated with a controlled amount of brain homogenate. The treated inoculum (still visible as a transparent biofilm with all the treatments done) was then scraped off and analysed for PrP<sup>res</sup>. Results were reproducible from one experiment to another, even when different brain homogenates were used. Moreover similar results were obtained on steel surfaces (data not shown).

In the absence of any decontamination procedure, normal brain inoculum yielded a PrP signal up to a dose

Treatments	Transmission rate (%)	Total death / total number	Disease duration in days (mean±sem)	Log reduction
NaOCl 20000 ppm, 20°C, 1 h	0%	0/8	>365	>5.6
NaOH 1N, 20°C, 1 h	0%	0/12	>365	>5.6
Autoclaving* 134°C, 18 min	60%	6/10 (7/10) <sup>a</sup>	197 (86)§	4.4-5
Autoclaving† 134°C, 18 min	0%	0/11	>365	>5.6
Enzymatic Cleaner+autoclaving‡ 0.8%, 43°C, 5 min/121°C, 20 min	10%	1/10 (4/10) <sup>a</sup>	242	~5
Enzymatic Cleaner 0.8%, 43°C, 5 min	100%	10/10	143 (12)§	~3.5
Alkaline Cleaner 1.6%, 43°C, 15 min	0%	0/11	>365	>5.6
Peracetic acid 0.25%, 55°C, 12 min	100%	12/12	155 (60)§	~3.5
Phenolic disinfectant 5%, 20°C, 30 min	0%	0/11	>365	>5.6
VHP 1.5 mg/L, 25°C, 3 h	33%	4/12	170 (33)§	~4.5
Enzymatic cleaner +VHP 0.8%, 43°C, 5 mins / 1.5 mg/L, 25°C, 3 h	0%	0/11	>365	15.6

\*Wires were placed on support during autoclaving at 134°C. †Wires were immersed in water during autoclaving at 134°C. ‡After the enzymatic cleaner treatment, wires were immersed in water and autoclaved at 121°C. §Data are mean (SE). At the end of the study, a few asymptomatic animals were positive for PrP<sup>res</sup> and incorporated as a positive transmission.

**Table 2: Effect of various treatments on contaminated wires**



**Figure 1:** Western-blot analysis of PrP<sup>res</sup> adsorbed on glass slide surfaces and treated with different chemical formulations and proteinase K concentrations.

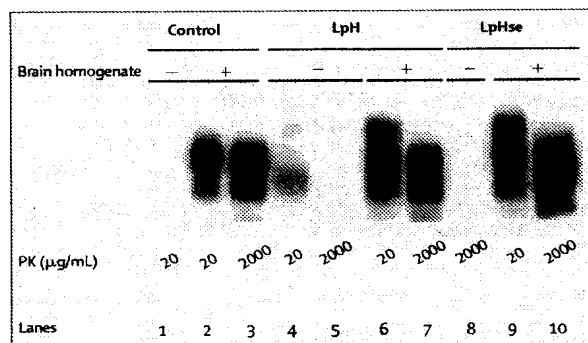
All lanes correspond to the analysis of 75 µg of brain equivalent. (A) compares VHP treatment versus control (untreated biofilm). (B) shows treatment at 43°C with water, alkaline cleaner at 0.16%, and equivalent concentrations of alkali (0.006N KOH or NaOH).

of 2 µg/mL of proteinase K. In infected brain, the PrP<sup>res</sup> persisted even at the proteinase K dose of 2 mg/mL (figure 1A, control). Application of the VHP procedure led to an increase of the PrP<sup>res</sup> signal in the absence of proteinase K treatment; however, this PrP<sup>res</sup> species was rendered fully proteinase K sensitive as shown by the disappearance of the PrP<sup>res</sup> signal at the lowest dose of 2 µg/mL PK (figure 1A).

The alkaline cleaner (composed of KOH with detergents) combined a removal effect of PrP<sup>res</sup> with a sensitisation to proteinase K (figure 1B, H<sub>2</sub>O and alkaline cleaner panels—lanes 5 to 8). The PrP<sup>res</sup> signal disappeared completely at a proteinase K dose of 20 µg/mL. Compared with unformulated alkali at the same concentrations (0.006N of KOH or NaOH, equal to the concentration of 0.16% of alkaline cleaner used in the in-vitro experiment), PrP<sup>res</sup> signal was not removed with 20 µg/mL of proteinase K following alkali treatment, whereas it was eliminated with 8 µg/mL of proteinase K for the alkaline cleaner (figure 1B). At the recommended concentration used in the in-vivo study (1.6%), no signal was observed (data not shown). Moreover, this effect was observed at 43°C (recommended temperature), whereas no effect on PrP was seen at lower temperatures (below 25°C; data not shown).

The phenolic compounds of Environ LpH degraded the brain layer coated on glass slides into a sticky gum incompatible with further experiments. Thus, we had to undertake the decontamination procedure for the in-vitro analysis of this formulation on brain homogenates. Both Environ LpH (the formulation under test) and LpHse (a distinct phenolic formulation) increased the resistance of PrP<sup>c</sup> to proteinase K at the dose of 20 µg/mL (lanes 4 and 1 in figure 2; plus data not shown). At the same dose of 20 µg/mL proteinase K, Environ LpH and LpHse treatments led to the appearance of an aggregated form of PrP<sup>res</sup> (figure 2, lanes 6 and 9 vs 2, note the band of higher molecular weight). At the higher proteinase K dose of 2000 µg/mL, no difference was seen between each treatment and the control (figure 2, lanes 7 and 10 vs 3).

Immunohistochemical analyses of the brains of wire-implanted and diseased hamsters showed widespread PrP<sup>res</sup> deposition at the terminal stage of disease in the same regions as in hamsters inoculated with homogenates (figure 3, B and C). Additionally, the area directly adjacent to the wire was heavily stained with PrP<sup>res</sup> (figure 3, B). In the brains of hamsters that developed disease after implantation of wires treated with procedures allowing only partial decontamination (enzymatic cleaner alone—figure 3, D), the PrP<sup>res</sup> pattern was broadly similar to that of the control hamsters implanted with untreated wires (figure 3, B). Hamsters implanted with treated wires showing no clinical signs of neurological disease were killed at 400 days post-inoculation to verify the absence of incipient PrP<sup>res</sup>.



**Figure 2:** Effect of phenolic formulations Environ LpH and LpHse on PrPc and PrPsc

PrP<sup>sc</sup> purified from the treated sample was treated with increasing amounts of proteinase K. All lanes correspond to the analysis of 200 µg of brain homogenate. Lanes 1, 4, 5, and 8 are non-infected brain homogenate; lanes 2, 3, 6, 7, 9, and 10 are scrapie brain homogenate.

deposition. No PrP<sup>sc</sup> deposits were seen in hamsters implanted with wires treated with either the reference NaOH treatment or a combination of enzymatic cleaner and VHP (figure 3, E and F) as observed in hamsters implanted with negative control wires (figure 3, A).

## Discussion

Using WHO reference treatments<sup>9</sup> as decontamination controls, we confirmed the effectiveness of 1N NaOH and 20 000 ppm NaOCl to decontaminate wires coated with hamster-adapted scrapie, as had been previously shown for tissue suspensions.<sup>28</sup> We also confirmed that inactivation by autoclaving at 134°C for 18 min was only complete when the wires were immersed in water: incomplete inactivation of wires placed in the autoclave on a dry support underlines the protective or fixing effect which can occur when dried material is autoclaved.<sup>23</sup> Exposure to formulated peracetic acid at 55°C, currently recommended as a replacement for glutaraldehyde for the decontamination of endoscopes, yielded a 3.5 log reduction of infectivity, in accord with data reported by other researchers,<sup>24,25</sup> and in the same range as decontamination using an enzymatic cleaner. The use of this cleaner in the presence of VHP or with conventional autoclaving at 121°C, produced nearly complete decontamination. Further, the alkaline cleaner and phenolic disinfectant Environ LpH were also shown to be effective. The implication of these findings are as follows. First, VHP is a non-corrosive gas disinfectant and steriliser that can be used on fragile or inaccessible surfaces of complex instruments—for decontamination of fragile devices such as endoscopes, we propose the use of the alkaline cleaner on wet instruments followed (after drying) by a terminal VHP treatment. Second, the combination of the enzymatic cleaner with conventional 121°C autoclaving is of special interest for use in facilities that cannot support an extended 134°C cycle. Finally, the alkaline cleaner, because of its formulation

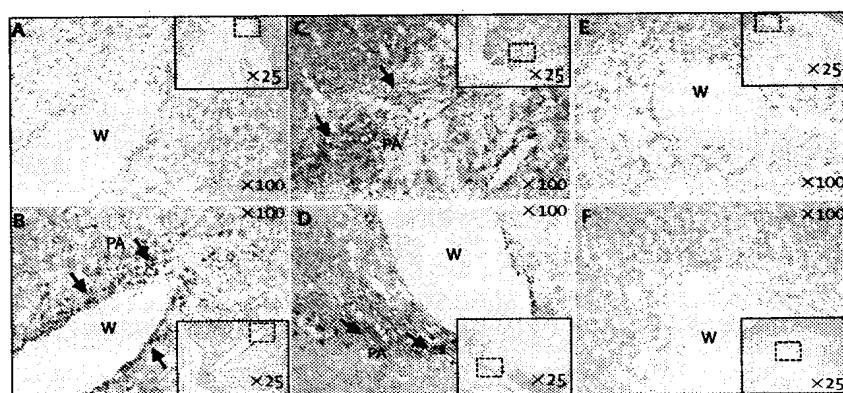
with a moderate alkali concentration, is less corrosive than currently recommended chemical decontamination procedures and could also be amenable for use on fragile instruments. Environ LpH may be useful for environmental decontamination of large surfaces.

Biochemical PrP<sup>sc</sup> analysis highlighted three different mechanisms of action for the decontamination procedures under test.

The alkaline cleaner reduced PrP<sup>sc</sup> amounts and increased PK sensitivity, similar to findings in studies involving the use of brain homogenates.<sup>26</sup> The higher efficiency of the alkaline cleaner compared to an equivalent concentration of pure alkaline solution (NaOH or KOH) was probably due to a combination of denaturation by the alkali and removal by the detergents contained in the formulation. Moderate heating (43°C) increased the efficiency of alkaline treatment (data not shown).

VHP showed a different effect: a paradoxical increase of PrP<sup>sc</sup> in the absence of proteinase K treatment, but a disappearance of PrP<sup>sc</sup> at even the lowest concentration of proteinase K (similar to uninfected controls). In gaseous form, hydrogen peroxide seems to alter the structure of the protein in such a way that normally inaccessible epitopes of the molecules are exposed and become immunoreactive, resulting in both enhanced immunoreactivity and increased proteinase K sensitivity. VHP has been shown in other studies to break down proteins into smaller peptides (Antloga K, McDonnell G, unpublished data).

A third type of effect was seen with Environ LpH. This product increased both PrPc and PrP<sup>sc</sup> aggregation, leading to a slightly increased proteinase K resistance of PrPc and the appearance of a high molecular weight band for PrP<sup>sc</sup>, which was neither eliminated nor



**Figure 3:** PrP immunostaining of the brains of hamsters implanted with contaminated wires. W=wire, PA=PrP<sup>sc</sup> accumulation. Panels show magnifications of the areas within the dotted frames in the insets. No immunoreactivity in brain from an uninfected animal (A). PrP<sup>sc</sup> immunostaining present in the brains of an animal implanted with a wire exposed to a 1×10<sup>4</sup> dilution of brain homogenate (B), an animal inoculated intracerebrally with 50 µL of brain homogenate (C), and an animal implanted with a wire treated by an enzymatic cleaner solution (D). No immunoreactivity was detected in the brain of an animal implanted with a wire treated with enzymatic cleaner plus VHP (E) or with 1N NaOH (F). Detection of PrP<sup>sc</sup> was visualised with biotinylated-SAF 32.

rendered proteinase K sensitive. Furthermore, both Environ LpH and LpHse had a similar effect on PrP even though one removed infectivity, whereas the other did not.<sup>27</sup> These results suggest either a decontaminating effect relying on a molecular entity other than PrP, or an effect on PrP<sup>res</sup> that cannot be detected by the type of immunological analysis performed in this study. In any case, our data show that the assessment of decontaminant efficacy should not rely solely on western-blot analyses.

Our present study paves the way for testing innovative decontamination procedures with use of an experimental method designed to mimic medical or surgical decontamination practices, and can be used in future studies of other prion strains such as sCJD and vCJD. Our data highlight different possible molecular mechanisms leading to the decontamination effect and warrant further investigations of these and other formulations. A better understanding of these mechanisms might facilitate the development of biochemical methods for higher throughput screening of useful decontamination and other inactivation procedures. Of more immediate importance, we described three procedures suitable for the decontamination of fragile surgical instruments, one of which may also be useful for medical devices containing electronic or video components (eg, endoscopes, laparoscopes).

#### Contributors

J P Deslys, E Comoy, and G McDonnell were responsible for design and management of this study. G Fichet undertook the biochemical analyses and C Duval the in-vivo study. K Antloga prepared and assisted with the wire preparation and decontamination. C Dehen did the in-vitro study. A Charbonnier undertook the immunohistochemical analyses. C I Lasmézès, G Fichet, E Comoy, G McDonnell, P Brown, and J P Deslys drafted the manuscript.

#### Conflict of interest statement

K Antloga and G McDonnell are employees of the manufacturer who provided the chemical components (Steris). All authors had full access to all data and had responsibility for the decision to submit for publication.

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